

## ORIGINAL ARTICLE

# Sevoflurane attenuates systemic inflammation compared with propofol, but does not modulate neuro-inflammation

## *A laboratory rat study*

Beatrice Beck-Schimmer\*, Lukas Baumann\*, Tanja Restin, Philipp Eugster, Melanie Hasler, Christa Booy and Martin Schläpfer

**BACKGROUND** Septic encephalopathy is believed to be a result of neuro-inflammation possibly triggered by endotoxins, such as lipopolysaccharides (LPS). Modulation of the immune system is a property of volatile anaesthetics.

**OBJECTIVE** We aimed to investigate the systemic and cerebral inflammatory response in a LPS-induced sepsis model in rats. We compared two different sedation strategies, intravenous propofol and the volatile anaesthetic sevoflurane, with the hypothesis that the latter may attenuate neuro-inflammatory processes.

**DESIGN** Laboratory rat study.

**SETTING** Basic research laboratories at the University Hospital Zurich and University Zurich Irchel between August 2014 and June 2016.

**PATIENTS** A total of 32 adult male Wistar rats.

**INTERVENTIONS** After tracheotomy and mechanical ventilation, the anaesthetised rats were monitored before sepsis was induced by using intravenous LPS or phosphate-buffered saline as control. Rats were sedated with propofol ( $10 \text{ mg kg}^{-1} \text{ h}^{-1}$ ) or sevoflurane (2 vol%) continuously for 12 h.

**MAIN OUTCOME MEASURES** Systemic inflammatory markers such as cytokine-induced neutrophil chemo-attractant protein 1, monocyte chemo-tactic protein-1 and IL-6 were determined. The same cytokines were measured in brain

tissue. Cellular response in the brain was assessed by defining neutrophil accumulation with myeloperoxidase and also activation of microglia with ionised calcium-binding adaptor molecule-1 and astrocytes with glial fibrillary acidic protein. Finally, brain injury was determined.

**RESULTS** Animals were haemodynamically stable in both sedation groups treated with LPS. Blood cytokine peak values were lower in the sevoflurane-LPS compared with propofol-LPS animals. In brain tissue of LPS animals, chemo-attractant protein-1 was the only significantly increased cytokine ( $P = 0.003$ ), however with no significance between propofol and sevoflurane. After LPS challenge, cerebral accumulation of neutrophils was observed. Microglia activation was pronounced in the hippocampus of animals treated with LPS ( $P = 0.006$ ). LPS induced prominent astrogliosis ( $P < 0.001$ ). There was no significant difference in microglia or astrocyte activation or apoptosis in the brain between sevoflurane and propofol.

**CONCLUSION** We have shown that systemic attenuation of inflammation by the volatile anaesthetic sevoflurane did not translate into attenuated neuro-inflammation in this LPS-induced inflammation model.

**TRIAL REGISTRATION** Animal approval No. 134/2014, Veterinäramt Zürich.

Published online 28 July 2017

## Introduction

Sepsis is an overwhelming systemic inflammatory response to an infection and carries a high mortality and

morbidity.<sup>1,2</sup> Its prevalence is about 1 per 1000 people in industrialised countries<sup>3</sup> and it accounts for 10 to 50% of

From the Institute of Anaesthesiology, University Hospital Zurich (BBS, LB, TR, PE, MH, MS), Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland (BBS, LB, TR, CB, MS); and Department of Anaesthesiology, University of Illinois at Chicago, Chicago, Illinois, USA (BBS)

Correspondence to Martin Schläpfer, MD, MSc, Institute of Anaesthesiology, University Hospital Zurich, Raemistrasse 100, CH-8091 Zurich, Switzerland  
Tel: +41 44 635 5035; e-mail: martin.schlaepfer@uzh.ch

\*Beatrice Beck-Schimmer and Lukas Baumann contributed equally as first authors.

deaths in ICUs.<sup>4,5</sup> Septic encephalopathy is one of the most common complications of sepsis and often precedes multi-organ failure.<sup>6,7</sup> It probably results from neuro-inflammation, altered cerebral perfusion and imbalance of neurotransmitters.<sup>8</sup> It may present as any kind of neurological dysfunction and even lead to coma.<sup>9</sup> Abnormal electroencephalography patterns have been reported in more than 80% of septic patients.<sup>10</sup> It has been shown that septic encephalopathy is associated with poorer outcome in sepsis independent of other organ dysfunction.<sup>11</sup> Recent data indicate that long-term neurological impairment is common even after recovery from sepsis.<sup>12</sup> The importance of cerebral dysfunction in sepsis is underlined by the quick sepsis-related organ failure assessment score that aims to identify those suffering from an infection, who are prone to poor outcome, including cerebral dysfunction.<sup>13</sup>

The pathophysiology behind septic encephalopathy is unclear, and there is no targeted treatment, but it has been shown that both ischaemic and neuro-inflammatory processes trigger the development of septic encephalopathy during sepsis.<sup>14</sup> In-vivo models demonstrate that septic encephalopathy is accompanied by a disrupted blood–brain barrier<sup>15,16</sup> and that [lipopolysaccharides (LPS)] exposure induces an astroglial and microglial inflammatory response,<sup>17,18</sup> promoting transmigration of monocytes and neutrophils.<sup>19</sup> More importantly, LPS not only induces an inflammatory response in the brain, but also impairs cognitive function in rats and mice.<sup>20,21</sup>

Sevoflurane is a volatile anaesthetic and sedative, which reduces endothelial inflammatory response after LPS exposure *in vitro* and *in vivo*.<sup>22,23</sup> It also improves survival rate in septic rats and mice.<sup>24,25</sup>

The aim of this study was to assess neuronal inflammation in a LPS-induced sepsis model and to investigate any immuno-modulation of the inflammatory process by sevoflurane sedation. We chose to do this by first determining the release of pro-inflammatory cerebral cytokines such as chemoattractant protein-1 (CINC-1), monocyte chemotactic protein (MCP) and IL-6 and also the anti-inflammatory transforming growth factor beta 1 (TGF $\beta$ 1) in LPS-treated animals. We intended to assess their functional effect, namely recruitment of neutrophils as effector cells. Microglial (macrophage) activation was assessed by measuring ionised calcium-binding adaptor molecule-1 (Iba-1). To assess whether and how astroglial inflammatory response, defined as reactive astrogliosis, in rat brain tissue develops, we measured astrocytic activation through glial fibrillary acidic protein (GFAP). Finally, cerebral apoptosis was evaluated. Based on previous observations in non-neuronal tissue, we hypothesised that sevoflurane, compared with propofol, might have an attenuating effect on the accumulation of neutrophils as well as activation of microglia and astrocytes.

## Material and methods

### Ethics

The animal experiments were approved by the Animal Use and Care Committee of the Kanton Zurich (Veterinäramt des Kantons Zürich, Zollstrasse 20, 8090 Zurich, Switzerland, approval No. 134/2014, 1 July 2014). The trial was registered and given Animal approval No. 134/2014 by the Veterinäramt Zürich.

The animal experiments took place between 18 August 2014 and 12 November 2014. Molecular analyses followed between December 2014 and June 2016.

### Animals

All experiments were carried out on adult male Wistar rats (Charles River, Sulzfeld, Germany) weighing  $329 \pm 21$  g (corresponding to 9 to 11 weeks). Animals were allowed to adapt for 1 week prior to the experiments. They were housed in standard rat cages with a 12/12 h light/dark cycle and free access to food and water.

### Animal model and anaesthesia

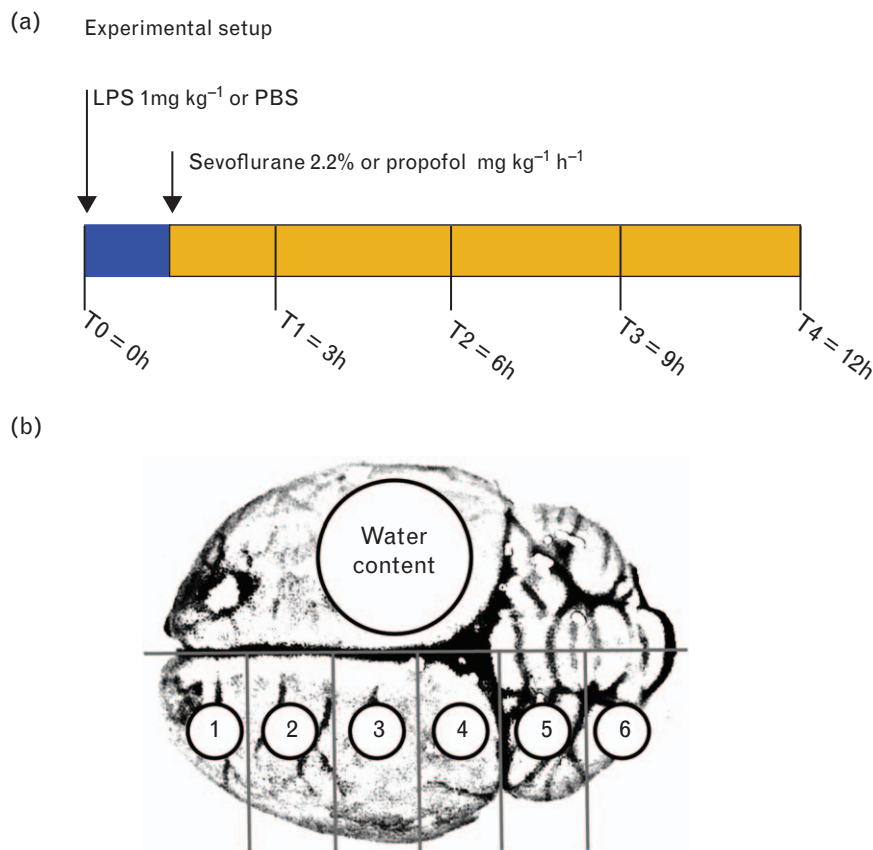
Despite its limitations, endotoxaemia is accepted as an in-vivo sepsis model. It allows good control of the degree of systemic inflammation without evoking animal death. To avoid any sepsis-induced vascular effects such as ischaemia, we chose a more haemodynamically stable model giving priority to the inflammatory component.

An overview of the experimental procedure is given in Fig. 1a. On the day of the experiment, blocks of two animals were randomly assigned to four intervention groups, with eight animals in each group:

- (1) propofol-LPS: endotoxaemia induced by LPS, sedation for 12 h with propofol
- (2) sevoflurane-LPS: endotoxaemia induced by LPS, sedation for 12 h with sevoflurane
- (3) propofol-PBS: sham procedure with PBS, sedation for 12 h with propofol
- (4) sevoflurane-PBS: sham procedure with PBS, sedation for 12 h with sevoflurane

Anaesthesia was induced with an intra-peritoneal injection of ketamine, midazolam and fentanyl ( $100$ ,  $3$  and  $0.015$  mg kg<sup>-1</sup>, respectively). The common carotid artery and external jugular vein were cannulated and a tracheotomy was performed. The lungs were mechanically ventilated (Servo Ventilator 300; Maquet, Solna, Sweden). The baseline ventilator settings were: positive end-expiratory pressure 3 cmH<sub>2</sub>O; peak inspiratory pressure 14 cmH<sub>2</sub>O; fraction of inspired oxygen 0.3; respiratory rate of 30 min<sup>-1</sup>, in line with previous experiments.<sup>26</sup> Anaesthesia was maintained by either sevoflurane (2.2%) or propofol (10 mg kg<sup>-1</sup> h<sup>-1</sup>). The presence of the toe-pinch reflex was sought, but animals had to be able to tolerate ventilation without distress. Depth of anaesthesia was tested every 30 min and in case of respiratory

Fig. 1



Experimental setup with time-line: after a single injection of lipopolysaccharides to induce endotoxaemia or phosphate-buffered saline (sham animals), male Wistar rats were continuously sedated for 12 h with either propofol (10 mg kg<sup>-1</sup> h<sup>-1</sup>) or sevoflurane 2.0 vol%. Blood samples were drawn every 3 h, brain tissue was obtained for histological and biochemical analyses. (a) The left brain hemisphere was cut into six sections and used for histological and biochemical analyses. The sections used for the individual analyses are described in the corresponding the 'Material and methods' section. Brain water content calculation was performed in the right hemisphere (b).

distress, an additional fentanyl bolus of 5 µg kg<sup>-1</sup> was given. After letting the animals adapt for 10 min, baseline venous and arterial blood samples were drawn for analyses that included blood gases. The blood samples taken were replaced in a 1:4 ratio with Ringer's solution (Ringerfundin; B.Braun, Melsungen, Germany), administered continuously at 5 ml kg<sup>-1</sup> h<sup>-1</sup> initially.<sup>27</sup> In response to the blood gas analysis, the respiratory rate was adjusted for normoventilation (CO<sub>2</sub> target range: 35 to 45 mmHg).

Endotoxaemia was induced by intravenous LPS 1 mg kg<sup>-1</sup> body weight from *Escherichia coli* (Sigma-Aldrich, Buchs, Switzerland), dissolved in 300-µl PBS (Kantonsapotheke Zurich, Zurich, Switzerland). Sham animals received PBS only.

Blood gas analyses (Epoc blood gas analyser; Alere, Waltham, Massachusetts, USA) and venous blood samplings were repeated every 3 h until the end of the experiment. Blood samples were placed in heparinised tubes, centrifuged at 4 °C and 8000 relative centrifugal

force (rcf), and plasma was stored at -80 °C. Animals were kept at 37 °C throughout the procedure, by using a heating pad. At the end of the experiment, animals were sacrificed under deep anaesthesia, and the brain was removed. The left hemisphere of the brain was sliced into six sections (Fig. 1b). Half of these were snap-frozen in liquid nitrogen and stored at -80 °C; the other half were stored in 4% formaldehyde (Kantonsapotheke Zurich) for at least 72 h, then formaldehyde was exchanged for ethanol (EtOH) 70%, and the sections were stored at 4 °C until paraffin embedding. The right hemisphere was used for brain water calculation.

#### Real-time PCR analysis of inflammatory markers in brain tissue

To isolate total RNA from brain tissue, the RNeasy Mini Kit (Qiagen, Basel, Switzerland) was used according to the manufacturer's protocol. Total RNA amounts and purity were determined by absorbance at 260 nm and the absorbance ratio at 260/280 nm by using NanoDrop ND

**Table 1** Primer sequences and probes used for real-time PCR

Gene	Primer sequence	Length of amplicon (nt)
<i>CINC-1</i>		
Forward	5' CAC ACT CCA ACA GAG CAC CA 3'	120
Reverse	5' TGA CAG CGC AGC TCA TTG 3'	
Probe # 49	5' CAG CCA CC 3'	
<i>MCP-1</i>		
Forward	5' AGC ATC CAC GTG CTG TCT C 3'	78
Reverse	5' GAT CAT CTT GCC AGT GAA TGA GT 3'	
Probe # 62	5' ACC TGC TG 3'	
<i>IL-6</i>		
Forward	5' CCC TTC AGG AAC AGC TAT GAA 3'	74
Reverse	5' ACA ACA TCA GTC CCA AGA AGG 3'	
Probe # 20	5' CTG GCT GG 3'	
<i>ICAM-1</i>		
Forward	5' TTC TGC CAC CAT CAC TGT GT 3'	95
Reverse	5' AGC GCA GGA TGA GGT TCT T 3'	
Probe # 62	5' ACC TGC TG 3'	
<i>TGFβ1</i>		
Forward	5' AAG GGC TAC CAT GCC AAC TT 3'	92
Reverse	5' TGG TTG TAG AGG GCA AGG AC 3'	
Probe # 116	5' GAG CCT GG 3'	

CINC-1, chemoattractant protein-1; ICAM-1; intercellular adhesion molecule-1; MCP-1, monocyte chemotactic protein-1; TGFβ1, transforming growth factor beta 1.

1000 (NanoDrop Technologies, Wilmington, Delaware, USA).

Reverse transcription and real-time quantitative PCR for CINC-1, MCP-1, IL-6, intercellular adhesion molecule-1 (ICAM-1) and TGFβ1 were performed as previously described.<sup>28</sup> Details of forward and reverse primers as well as the probes used can be found in Table 1.

### Protein extraction from neuronal tissue

Frozen sections of the frontal lobe (segment 1, Fig. 1b) of each specimen were homogenised in lysis buffer (1,4-dithiothreitol 100 mmol l<sup>-1</sup>; protease inhibitor 1:500; phosphatase inhibitor 2 + 3 1:100) from Sigma in Hank's Balanced Salt Solution (Thermo Fisher Scientific, Rockford, Illinois, USA). Ceramic beads (MagNA Lyser Green Beads; Roche Diagnostics, Mannheim, Germany) of a diameter of 1.4 mm were added to the tissue and homogenised by using Precellys Tissue Homogeniser (Bertin Corp., Rockville, Maryland, USA) for 20 s at 5500 rpm. The lysate was then centrifuged at 4 °C and 300 rcf for 10 min and the supernatant collected. Samples were stored at -20 °C until further analysis was performed.

### ELISAs

ELISAs for the inflammatory mediators CINC-1 (R&D Systems, Abingdon, United Kingdom), MCP-1 (BD Bioscience, Allschwil, Switzerland) and IL-6 (R&D Systems) were performed in serum (at 0, 3, 6, 9 and 12 h) and brain tissue lysate according to the manufacturer's protocol. To determine the protein concentration of brain tissue lysates, a detergent compatible protein assay

(Bio-Rad Laboratories AG, Cressier, Switzerland, Catalogue No. 500-0114) was performed as described.

The results from brain tissue lysates were calculated in pg ml<sup>-1</sup> and were corrected for the amount of protein in the lysate (divided by the protein content in the solution in mg ml<sup>-1</sup>). The final results are shown as pg cytokine per mg brain protein.

### Formalin fixed, paraffin embedded brain sections

Before embedding in paraffin, the brain tissue was dehydrated by using a tissue processor (Microm STP 120; Thermo, Reinach, CH, Basel, Switzerland) for a total of 17 h with 3 h 70% EtOH, 3 h 80% EtOH, 2 h 90% EtOH, 2 h 96% EtOH, 2 h 100% EtOH, 2 h xylene and 3 h paraffin. In the next morning, the tissue was embedded by using a paraffin embedding system (Mediate TBS 88; Dietikon, CH, Basel, Switzerland).

### Immuno-histochemistry

Paraffin-embedded brain tissue (the 'Material and methods' section, Fig. 1b) was cut into 5-μm thick coronal sections by using a Zeiss Hyrax M55 microtome (Carl Zeiss, Jena, Germany). Sections mounted onto Polysine microscope slides (VWR International, Leuven, Belgium) were dried on a slide warmer at 37 °C overnight.

To quantify the severity of neuro-inflammation, markers for neutrophils [myeloperoxidase (MPO), GFAP and Iba-1] were used. After removing paraffin in 3 × 5 min Xylene (Sigma), 2 × 5 min in 100% EtOH, 2 × 5 min in 95% EtOH and 1 × 5 min in 75% EtOH, the slides were washed three times in PBS.

For Iba-1 and MPO, but not for GFAP staining, heat-induced epitope retrieval in citrate buffer (1.8 mmol l<sup>-1</sup> citric acid, 8.2 mmol l<sup>-1</sup> sodium citrate; both from Sigma) was undertaken by using a Histos Pro microwave (Milestone SRL, Sorisole, Italy). All sections were blocked with 5% bovine serum albumin (BSA) solution (KPL, Gaithersburg, Maryland, USA) in PBS for 1 h at room temperature (RT). Sections were allowed to incubate with the primary antibody overnight at 4 °C: Iba-1 goat anti-rat antibody (Abcam; Catalog No. ab5076; 1:500), MPO rabbit anti-rat antibody (Abcam; Catalog No. ab9535; 1:50) and GFAP rabbit anti-rat (Sigma; Catalog No. G9269; 1:100). After washing in PBS, slides were incubated with the corresponding secondary antibody Alexa Fluor 488 donkey anti-goat IgG (Thermo Fisher Scientific; Catalog No. A-11055; 1:500) or Alexa Fluor 568 goat anti-rabbit IgG (Abcam; 1:500) and with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) 1:1000 (Roche) for 1 h at RT. All antibodies were diluted in 0.5% BSA in PBS solution. In a final step, all slides were mounted with fluorescent mounting medium (DAKO; Dako North America, Carpinteria, California, USA) or ProLong Gold anti-fade reagent (Thermo Fisher Scientific).



### Image processing and cell counting

GFAP and Iba-1 sections were evaluated by using a Leica DM5500 B microscope (Leica Microsystems, Heerbrugg, Switzerland) and image capture was performed by using a DFC 360 FX digital camera (Leica Microsystems). Images of the hippocampal dentate gyrus and retrosplenial cortex region (RSC) were taken at random at 20× magnification and then processed by using image analysis software (ImageJ 1.43; NIH, Bethesda, Maryland, USA). An identical threshold of the obtained image stacks was set, images were binarised and positive cells counted with the 'analyse particles tool' in ImageJ (Supplementary Information 1, <http://links.lww.com/EJA/A130>). The area of interest in the dentate gyrus was defined by outlining the polymorphic cell layer by using the DAPI co-staining. For each slide, up to four images per examined region were captured.

To visualise MPO stained brain sections, tile images with 20× magnification were taken by using an Axio Scan Z1 Slide Scanner (Carl Zeiss) and automatically merged by using ZEN 2.0 (Carl Zeiss) software. MPO immuno-reactive cells in the whole brain section area were automatically counted by using ImageJ as described above. Each identified particle was then manually verified to be a neutrophil. Then, the number of MPO positive cells per square millimetre (cells mm<sup>-2</sup>) was calculated and divided by the size of the analysed brain area. All images were post-processed and analysed in a blinded manner.

### Brain water content

The right hemisphere was used for brain water content calculation. The brain wet weight was determined immediately after removal. The hemispheres were dried for 72 h at 60 °C in the oven and the dry weight was assessed. Brain water content was calculated as follows: water [%] = 100 × (wet weight – dry weight)/wet weight.

### Determination of apoptotic cells

Neuronal apoptosis was evaluated by using the In Situ Cell Death Detection Kit (Roche) following manufacturers' instructions. The terminal deoxynucleotidyl transferase-mediated desoxy-uradyl triphosphate nick end labelling (TUNEL) reaction labels free 3'-OH termini in an enzymatic reaction and detects double or single-stranded DNA breaks generated during apoptosis.

### Study endpoints

In summary, the following were measured: mean arterial pressure, base excess, systemic and brain inflammatory mediators CINC-1, MCP-1 and IL-6, neutrophils in brain tissue, activation of microglia Iba-1-positive cells, activation of astrocytes (GFAP expression), brain oedema formation and the degree of neuronal apoptosis.

### Statistical analysis

Data are presented as mean ± SD. Analyses were performed in GraphPad Prism 6.0 for Mac OS X (GraphPad Inc, La Jolla, California, USA). Comparisons between the various groups were assessed by one-way analysis of variance (ANOVA), when single measurements were analysed. Repeated measurements were analysed by two-way ANOVA. Each treatment group was compared with every other treatment group. To correct for multiple comparisons, a Tukey post-hoc test was performed. The significance level was defined as *P* less than 0.05.

## Results

### Vital signs and systemic inflammatory markers

Mean arterial pressure was affected neither by sepsis nor by the type of sedation (propofol-LPS: 93 ± 13 mmHg, sevoflurane-LPS: 88 ± 6 mmHg, propofol-PBS: 105 ± 9 mmHg, sevoflurane-PBS: 93 ± 9 mmHg, Fig. 2a). Blood oxygenation (PaO<sub>2</sub>: propofol-LPS: 153 ± 20 mmHg, sevoflurane-LPS: 156 ± 16 mmHg, propofol-PBS: 160 ± 7 mmHg, sevoflurane-PBS: 168 ± 12 mmHg, all *P* > 0.8) and ventilation (PaCO<sub>2</sub>: propofol-LPS: 48 ± 3 mmHg, sevoflurane-LPS: 45 ± 4 mmHg, propofol-PBS: 45 ± 3 mmHg, sevoflurane-PBS: 41 ± 3 mmHg, all *P* > 0.5) were similar.

Base excess after 12 h was comparable in both LPS groups (*P* = 0.77), but lower than in the corresponding control groups (propofol-PBS: 0.7 ± 2.2 mEq l<sup>-1</sup> vs. propofol-LPS: -7.3 ± 2.2 mEq l<sup>-1</sup>, *P* < 0.001; sevoflurane-PBS: -0.2 ± 4.3 mEq l<sup>-1</sup> vs. sevoflurane-LPS: -5.7 ± 2.7 mEq l<sup>-1</sup>, *P* = 0.003) (Fig. 2b).

To assess inflammatory markers caused by endotoxaemia, plasma levels of CINC-1, MCP-1 and IL-6 were determined. Concentration of plasma CINC-1 in animals of both LPS groups peaked at 3 and 12 h. The inflammatory reaction was stronger with propofol than with sevoflurane animals (3 h: propofol-LPS vs. sevoflurane-LPS: 532 ± 189 vs. 368 ± 34 ng ml<sup>-1</sup>, *P* = 0.004; 12 h: propofol-LPS vs. sevoflurane-LPS: 401 ± 305 vs. 99 ± 40 ng ml<sup>-1</sup>, *P* < 0.001, Fig. 2c).

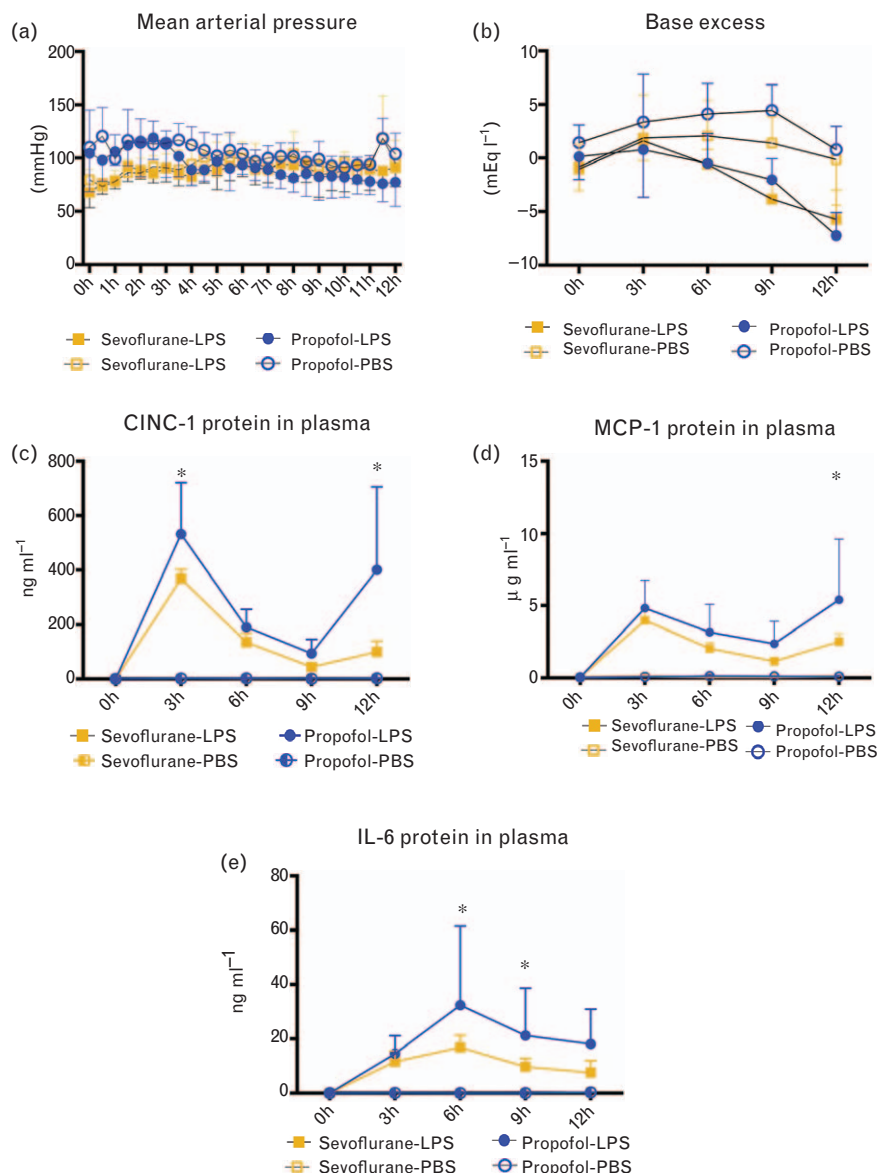
Similarly, a biphasic peak of MCP-1 (3 and 12 h) was observed in animals treated with LPS with a significant difference between the two sedative regimens at 12 h (propofol-LPS vs. sevoflurane-LPS: 5.3 ± 4.2 vs. 2.5 ± 0.5 µg ml<sup>-1</sup>, *P* < 0.001, Fig. 2d).

For IL-6 a single peak in LPS animals was found at 6 h. This was more pronounced in the propofol-LPS than the sevoflurane-LPS group (32 ± 29 vs. 17 ± 4 ng l<sup>-1</sup>, *P* = 0.003, Fig. 2e).

### mRNA and protein expression of inflammatory markers in brain tissue in sepsis

mRNA of the inflammatory mediators CINC-1, MCP-1 and IL-6 was upregulated after LPS injection and was

Fig. 2



Haemodynamic stability was verified in lipopolysaccharide and in sham (phosphate-buffered saline) animals under both sedative regimens with either propofol or sevoflurane (a). Acidosis was comparable in the two lipopolysaccharide groups (b). The systemic inflammatory markers cytokine-induced neutrophil chemo-attractant protein-1 (c), monocyte chemo-tactic protein-1 (d) and IL-6 (e) were increased at defined time-points in lipopolysaccharide animals and successfully attenuated in endotoxaemic animals sedated with sevoflurane. \* $P$  less than 0.05 propofol-lipopolysaccharide vs. sevoflurane-lipopolysaccharide.

similar in both sevoflurane-LPS and propofol-LPS groups (Fig. 3). Only the protein level of CINC-1 was increased after LPS with no significant difference between the anaesthetics used (Fig. 4).

#### Activation of microglia in sepsis

In the dentate gyrus area of the hippocampus, the amount of Iba-1 positive cells was lower in PBS compared with LPS animals (propofol-PBS vs. propofol-LPS:  $55.1 \pm 26.4$  vs.  $103.7 \pm 27.4$  cells mm<sup>-2</sup>,  $P = 0.006$ ; sevoflurane-PBS vs. sevoflurane-LPS  $55.2 \pm 18.8$  vs.  $123.9 \pm$

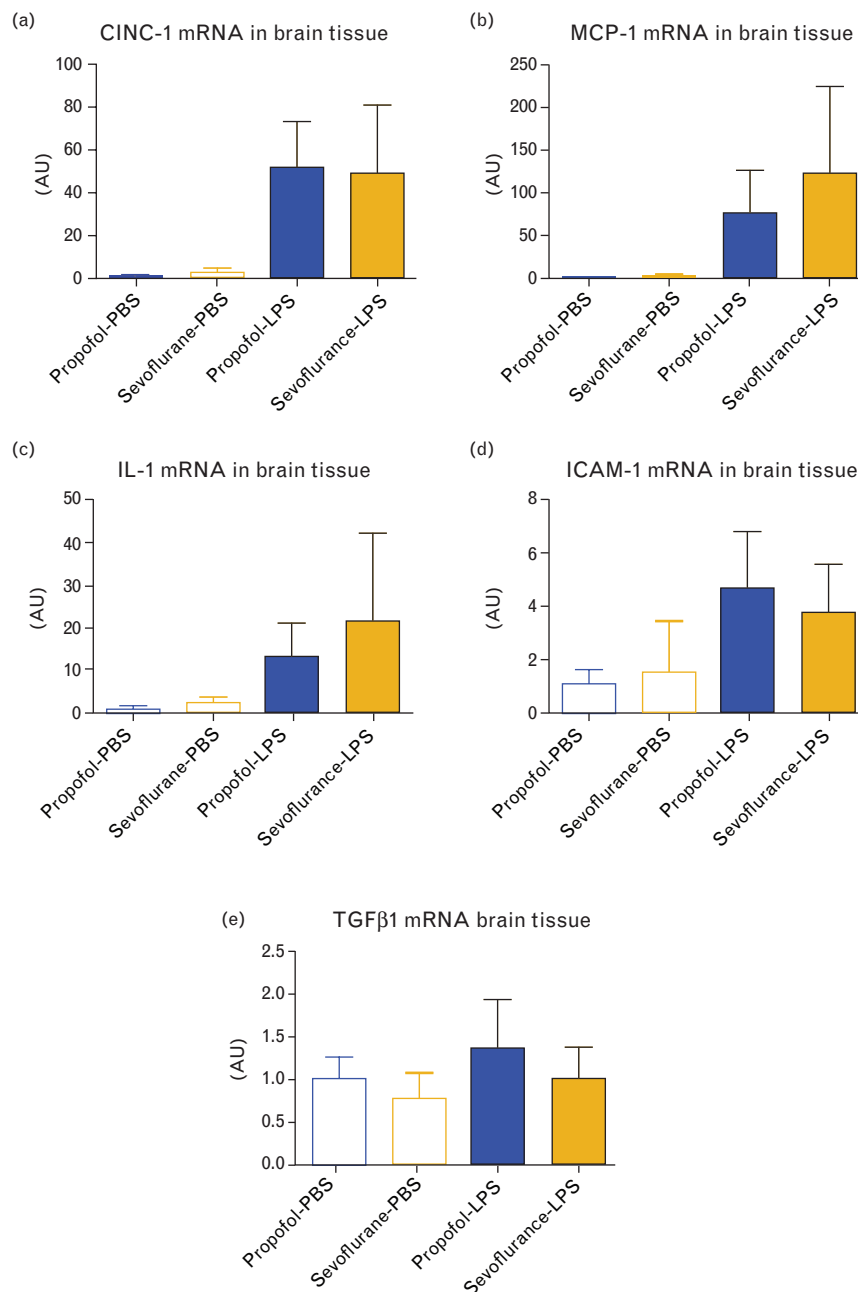
$34.2$  cells mm<sup>-2</sup>  $P < 0.001$ ). No effect of the type of sedative was observed in propofol-LPS vs. sevoflurane-LPS animals ( $P = 0.44$ , Fig. 5a to e).

Microglia in the cortex (RSC), assessed by Iba-1 positive cells, was not altered in LPS animals nor by the type of sedation.

#### Activation of astrocytes in sepsis

Astroglisis, assessed by positively GFAP staining in the hippocampal dentate gyrus region, was increased in

Fig. 3



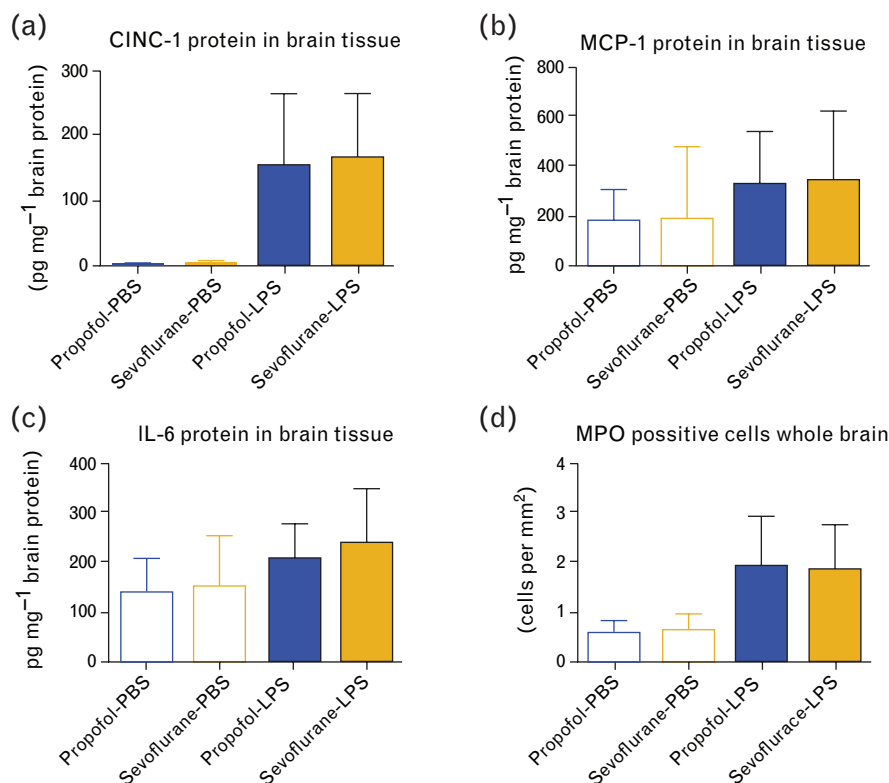
Brain mRNA of cytokine-induced neutrophil chemo-attractant protein 1 (a), monocyte chemo-tactic protein-1 (b), IL-6 (c), intercellular adhesion molecule-1 (d) and transforming growth factor  $\beta$  1 (e) were determined in brain tissue of lipopolysaccharide and sham animals (phosphate-buffered saline), comparing propofol with sevoflurane anaesthesia. Although chemoattractant protein-1, monocyte chemotactic protein-1 and intercellular adhesion molecule-1 mRNA were clearly increased in endotoxaemic animals, the inflammatory mediators were not affected by sevoflurane compared with propofol sedation.

propofol-LPS and sevoflurane-LPS animals vs. propofol-PBS ( $323 \pm 64$  and  $370 \pm 105$  vs.  $150 \pm 49$  cells  $\text{mm}^{-2}$ , both  $P < 0.001$ ). However, no difference with respect to the type of sedation in the two sepsis groups was observed ( $P = 0.57$ , Fig. 6a to e). In the cortical region, almost no activated astrocytes in LPS animals were found (figure not shown).

#### Impact of sepsis on blood–brain barrier

The blood–brain barrier function was indirectly assessed by determination of brain water. Brain water content was similar in control animals and in animals treated with LPS (propofol-PBS vs. propofol-LPS:  $79.71 \pm 0.27$  vs.  $79.53 \pm 0.33\%$ ,  $P = 0.70$  and sevoflurane-PBS vs. sevoflurane-LPS:  $79.67 \pm 0.41$  vs.  $79.16 \pm 0.29\%$ ,  $P = 0.05$ ).

Fig. 4



Brain protein levels of cytokine-induced neutrophil chemo-attractant protein 1 (a), monocyte chemotactic protein-1 (b), IL-6 (c) were determined in brain tissue of lipopolysaccharide and sham animals (phosphate-buffered saline), comparing propofol with sevoflurane anaesthesia. Chemoattractant protein-1 was increased in lipopolysaccharide animals compared with the sham groups, monocyte chemotactic protein-1 and IL-6, however, were not. The amount of neutrophil granulocytes in brain tissue, assessed by myeloperoxidase staining (d) was more accentuated in the lipopolysaccharide groups, however, they were not affected by sevoflurane compared with propofol sedation in the endotoxaemic animals.

Also, there was no significant difference between the two LPS groups ( $P = 0.22$ ).

#### Impact of sepsis on cerebral cell injury

By using TUNEL staining, no apoptotic cells were observed in the hippocampal and cortical areas of the animals after 12 h (the 'Material and methods' section, Fig. 1b, data not shown).

#### Discussion

The current study demonstrates enhanced expression of cytokines in the blood and brain of animals treated with LPS. After 12 h of sepsis, neutrophils were recruited into brain tissue in parallel with activation of macrophages (microglia) and an increase in astrogliosis, predominantly located in hippocampal tissue. No structural brain damage such as apoptosis or necrosis was observed. Although sevoflurane successfully reduced inflammatory mediators in the blood, endotoxin-induced neuro-inflammation remained.

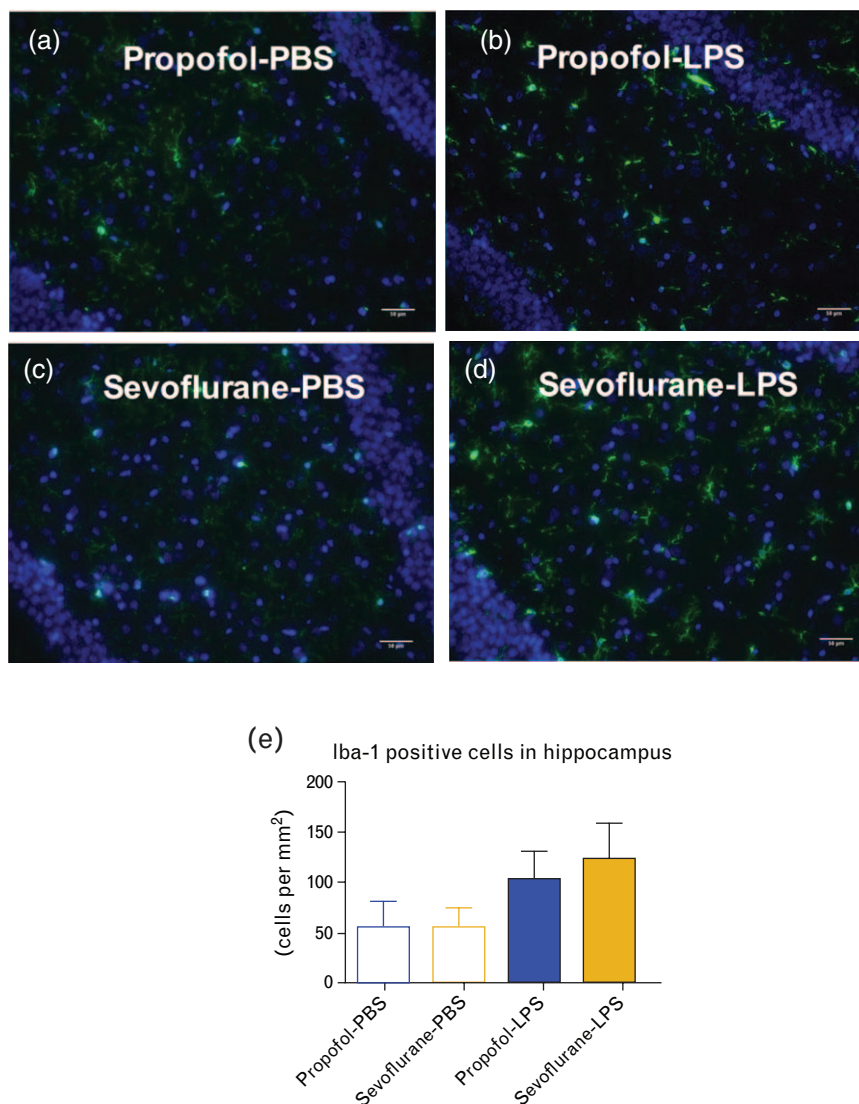
Septic encephalopathy is common in severe inflammation and is regarded as a serious complication<sup>9–11</sup>

with poorly understood pathogenesis and a lack of treatment options. Intravenous LPS administration induces systemic inflammation<sup>29</sup> and does affect the central nervous system (CNS).<sup>17,30</sup> We considered it a useful model for investigating neuro-inflammation in sepsis. Given that sevoflurane has a well known anti-inflammatory action during sepsis,<sup>22–25</sup> we were interested in exploring the effect of the volatile anaesthetic on neuronal inflammation after LPS exposure in rats.

The three inflammatory mediators CINC-1, MCP-1 and IL-6 do not only play a crucial role in endotoxaemia, but are also activated during inflammatory processes in the brain. CINC-1 (=CXCL1) is a well known and highly efficient chemo-attractant for neutrophils.<sup>31</sup> Its production in astrocytes is induced following neuronal injury,<sup>32</sup> and it contributes to neutrophil invasion into the brain<sup>33</sup> and to demyelination.<sup>34</sup> MCP-1 is a key attractor of monocytes and macrophages to sites of inflammation.<sup>35</sup> It is induced in brain tissue after systemic LPS-challenge.<sup>36</sup> Three facts, namely that MCP-1 (=CCL2) receptors are expressed by astrocytes,<sup>37</sup> neurons<sup>38</sup> and



Fig. 5

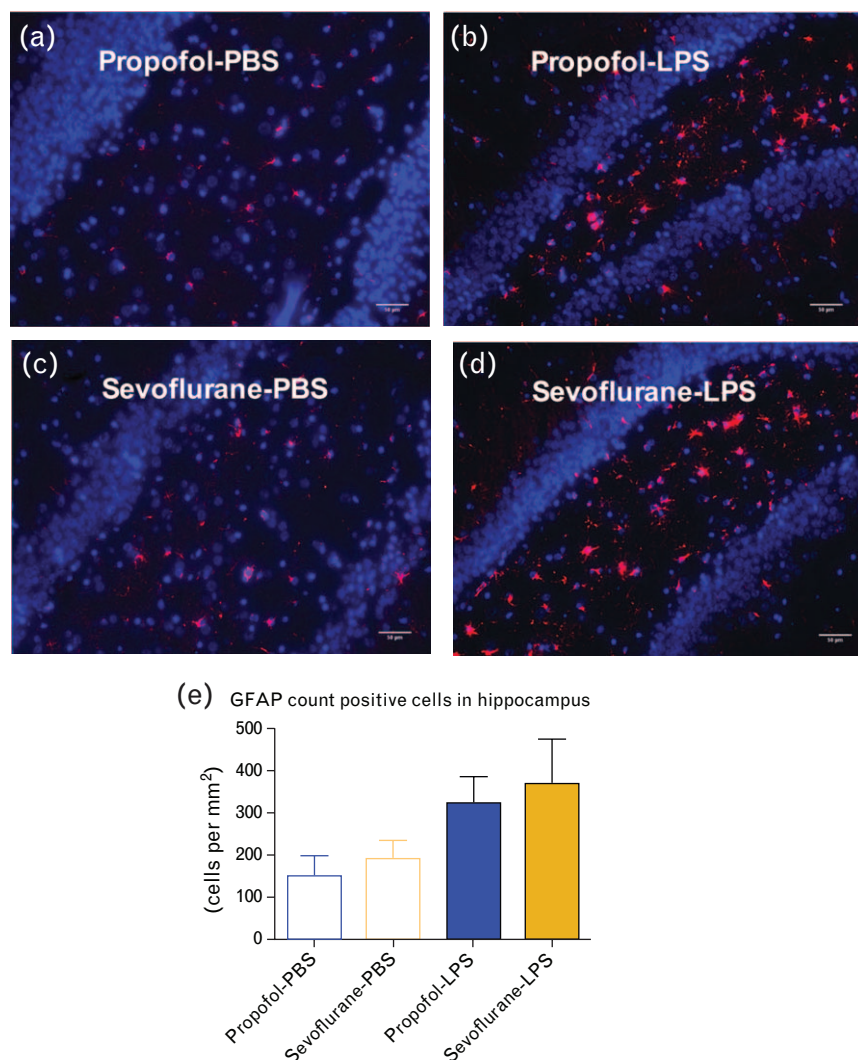


Activation of microglia, identified by ionised calcium-binding adapter molecule 1, was found in the dentate gyrus region of the hippocampus in lipopolysaccharide animals compared with the sham groups (phosphate-buffered saline), but was not influenced by the type of sedation propofol vs. sevoflurane (a to e).

microglia,<sup>39</sup> and that attenuation of MCP-1 was shown to be beneficial in sepsis in general,<sup>40</sup> and that severity of neuro-inflammation was reduced in MCP-1-knockout mice in endotoxin injury,<sup>41</sup> all indirectly underline the importance of MCP-1 as a sustaining factor for neuro-inflammation. MCP-1 contributes to blood–brain barrier disruption. It is not only secreted by immune cells, but also by neurons, astrocytes, microglia and brain endothelial cells.<sup>42</sup> IL-6 appears to be a predictor for mortality in septic patients.<sup>43</sup> In the brain IL-6 accumulates in experimental sepsis.<sup>44</sup> Its abundance in the brain, appears to be associated with severe cognitive impairment<sup>45</sup> and neuronal loss.<sup>46</sup>

In the proposed model, increased systemic levels of the pro-inflammatory mediators CINC-1, MCP-1 and IL-6 were attenuated in the presence of sevoflurane. This is in line with previous in-vivo and in-vitro findings.<sup>28,47,48</sup> CINC-1 mRNA and protein as well as MCP-1 mRNA were significantly upregulated in brain tissue in sepsis.<sup>49–51</sup> However, despite the systemic attenuating effect of sevoflurane, no differences were observed between propofol and sevoflurane sedation. However, by limiting the time to sacrifice the animals to 12 h does not permit full orchestration of the inflammatory response to systemically applied LPS in the brain. Mediators measured in the blood peak as early as 2 h following

Fig. 6



Activation of astrocytes, identified by green fibrillary acidic protein, was found in the dentate gyrus region of the hippocampus in lipopolysaccharide animals compared with sham groups (phosphate-buffered saline), but was not influenced by the type of sedation propofol vs. sevoflurane (a to e).

LPS, whereas the same mediators are discovered at a later time point in neuronal tissue with a peak between 8 and 12 h.<sup>52</sup> Markers of microglia activation are observed 4 to 6 h after LPS challenge, and they remain detectable for up to 12 h.<sup>52</sup> Activated GFAP-positive astrocytes are detectable as early as 6 h after LPS challenge<sup>53</sup> with a maximal response after 24 to 48 h.<sup>52</sup> Taking all these factors into account we concluded that the 12-h-time-point allows assessment of inflammatory mediators, reflecting a late phase of mediator expression. At the same time, determination of cellular activation of microglia and astrocytes by using Iba-1 and GFAP may be possible, which represents a rather early time-point for cellular changes. As a consequence, we cannot exclude that significant immuno-modulation by sevoflurane may be evident at a different time-point. A longer experiment

would have been desirable but would require a greater number of animals, something that may not accord with the aim of following the 3R principles in animal work.<sup>54</sup>

Of note is that the IL-6 protein expression in the brain is different than IL-6 mRNA with a significant difference between sham and LPS animals, whereas protein levels do not show this difference. This may be due to the later protein synthesis following mRNA expression.

In the current study, we did not focus on cytokine regulation alone, but rather aimed to cover a more complete picture of the neuro-inflammatory response, especially on a cellular level. Although in our model overall neutrophil count – assessed by MPO positive cells – was low, we found a significantly increased number of MPO positive cells in LPS animals. This correlates with

elevated mRNA and protein levels of CINC-1 and increased ICAM-1 mRNA expression. An additional group of inflammatory cells, microglia, are resident in the brain and represent a group of major active immune cells in the CNS.<sup>55</sup> Persistent activation of microglia may ultimately result in neuro-degeneration.<sup>56</sup> In our study, we found an increase in Iba-1 immuno-stained cells in the hippocampus, the most vulnerable brain region during sepsis.<sup>51</sup> Astrocytes in addition to microglia are important as structural cells of the brain and are important contributors to the inflammatory response of the CNS.<sup>55</sup> A characteristic protein of the cells is GFAP, which can be upregulated in response to inflammation.<sup>57</sup> Our data show a significant increase in GFAP positive cells in the dentate gyrus after LPS challenge. This microglial and astroglial activation following injury is in line with previous reports and reviews.<sup>18,51,56,58</sup> Other researchers described involvement of hippocampal, microglial and astroglial cells at later time-points after LPS challenge, after 24 and 72 h, respectively.<sup>59,60</sup> This discrepancy might be due to different LPS concentrations, routes of administration (intra-peritoneal, intrathecal) or animal models (mice).

No structural damage of the brain in sepsis was found in our model. Neither induction of apoptosis, assessed by TUNEL-positive cellular staining nor alterations in brain water content, as a measure of brain oedema, could be demonstrated when comparing sham and LPS animals.

Like all research, our experimental approach has strengths and limitations. The animal model used is well established and generates reproducible data with regard to the inflammatory response.<sup>24,28</sup> By using continuous monitoring and sedation, mechanical ventilation, and a fluid resuscitation protocol, it was closer to an ICU setting than many other studies. Nevertheless, we are aware that a direct translation from animal data to a clinical setting is not possible for a number of reasons.<sup>61</sup> First, a single LPS challenge is not likely to mimic all aspects of the interactive inflammatory response of human sepsis.<sup>56,62</sup> Second, tissue evaluation at a single time-point does not provide details of development over time, neither does it help with the long-term neurological sequelae of sepsis.<sup>63</sup> Third, rather young male animals were used in this study. The effect of anaesthetics<sup>64</sup> and immune response may be age<sup>65,66</sup> and sex related<sup>67</sup> with older individuals being more prone to cognitive dysfunction.<sup>68</sup> Finally, with this first basic analysis of neuro-inflammation during endotoxaemia, cognitive impairment was not assessed in our animals. This is a complex task and clearly not within the expertise of our team.

## Conclusion

Our study confirms a systemic anti-inflammatory effect of sevoflurane compared with propofol in a model of LPS-induced sepsis in rats. There were no differences in markers of neuro-inflammation between the sevoflurane and propofol group within the first 12 h following LPS

injection. Further investigations, including analysis at multiple time points and long-term assessments of neurologic outcomes that include functional evaluation, are necessary to confirm these results *in vivo*, followed by clinical trials.

## Acknowledgements

Assistance with the study: none.

Financial support and sponsorship: the current study was supported by the University of Zurich ('Filling the Gap' research fund for young researchers; grant no. FTG-1415-005) from MS, and through institutional funding.

Conflicts of interests: none.

Presentation: preliminary data of this project have been presented at the Euroanaesthesia Congress 2016 in London, abstract number: 01AP06-1.

## References

- 1 Vincent JL, Marshall JC, Namendys-Silva SA, *et al.* Assessment of the worldwide burden of critical illness: the intensive care over nations (ICON) audit. *Lancet Respir Med* 2014; **2**:380–386.
- 2 Winters BD, Eberlein M, Leung J, *et al.* Long-term mortality and quality of life in sepsis: a systematic review. *Crit Care Med* 2010; **38**:1276–1283.
- 3 Moss M. Epidemiology of sepsis: race, sex, and chronic alcohol abuse. *Clin Infect Dis* 2005; **41** (Suppl 7):S490–S497.
- 4 Kaukonen KM, Bailey M, Suzuki S, *et al.* Mortality related to severe sepsis and septic shock among critically ill patients in Australia and New Zealand, 2000–2012. *JAMA* 2014; **311**:1308–1316.
- 5 Martin GS, Mannino DM, Eaton S, *et al.* The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med* 2003; **348**:1546–1554.
- 6 Papadopoulos MC, Davies DC, Moss RF, *et al.* Pathophysiology of septic encephalopathy: a review. *Crit Care Med* 2000; **28**:3019–3024.
- 7 Ziaja M. Septic encephalopathy. *Curr Neurol Neurosci Rep* 2013; **13**:383.
- 8 Tsuruta R, Oda Y. A clinical perspective of sepsis-associated delirium. *J Intensive Care* 2016; **4**:18.
- 9 Bolton CF, Young GB, Zochodne DW. The neurological complications of sepsis. *Ann Neurol* 1993; **33**:94–100.
- 10 Young GB, Bolton CF, Archibald YM, *et al.* The electroencephalogram in sepsis-associated encephalopathy. *J Clin Neurophysiol* 1992; **9**:145–152.
- 11 Frontera JA. Metabolic encephalopathies in the critical care unit. *Continuum* 2012; **18**:611–639.
- 12 Widmann CN, Heneka MT. Long-term cerebral consequences of sepsis. *Lancet Neurol* 2014; **13**:630–636.
- 13 Wang JY, Chen YX, Guo SB, *et al.* Predictive performance of quick Sepsis-related Organ Failure Assessment for mortality and ICU admission in patients with infection at the ED. *Am J Emerg Med* 2016; **34**:1788–1793.
- 14 Sonnevile R, Verdonk F, Rauturier C, *et al.* Understanding brain dysfunction in sepsis. *Ann Intensive Care* 2013; **3**:15.
- 15 Jeppsson B, Freund HR, Gimmon Z, *et al.* Blood–brain barrier derangement in sepsis: cause of septic encephalopathy? *Am J Surg* 1981; **141**:136–142.
- 16 Davies DC. Blood–brain barrier breakdown in septic encephalopathy and brain tumours. *J Anat* 2002; **200**:639–646.
- 17 Jeong HK, Jou I, Joe EH. Systemic LPS administration induces brain inflammation but not dopaminergic neuronal death in the substantia nigra. *Exp Mol Med* 2010; **42**:823–832.
- 18 Kinoshita PF, Yshii LM, Vasconcelos AR, *et al.* Signaling function of Na, K-ATPase induced by ouabain against LPS as an inflammation model in hippocampus. *J Neuroinflamm* 2014; **11**:218–1218.
- 19 Zhou H, Andonegui G, Wong CH, *et al.* Role of endothelial TLR4 for neutrophil recruitment into central nervous system microvessels in systemic inflammation. *J Immunol* 2009; **183**:5244–5250.
- 20 Sun J, Zhang S, Zhang X, *et al.* IL-17A is implicated in lipopolysaccharide-induced neuroinflammation and cognitive impairment in aged rats via microglial activation. *J Neuroinflamm* 2015; **12**:165.
- 21 Lee JW, Lee YK, Yuk DY, *et al.* Neuro-inflammation induced by lipopolysaccharide causes cognitive impairment through enhancement of beta-amyloid generation. *J Neuroinflamm* 2008; **5**:37.



- 22 Rodriguez-Gonzalez R, Baluja A, Veiras Del Rio S, *et al.* Effects of sevoflurane postconditioning on cell death, inflammation and TLR expression in human endothelial cells exposed to LPS. *J Transl Med* 2013; **11**:87.
- 23 Voigtsberger S, Lachmann RA, Leutert AC, *et al.* Sevoflurane ameliorates gas exchange and attenuates lung damage in experimental lipopolysaccharide-induced lung injury. *Anesthesiology* 2009; **111**: 1238–1248.
- 24 Schlapfer M, Piegeler T, Dull RO, *et al.* Propofol increases morbidity and mortality in a rat model of sepsis. *Crit Care* 2015; **19**:45.
- 25 Herrmann IK, Castellon M, Schwartz DE, *et al.* Volatile anesthetics improve survival after cecal ligation and puncture. *Anesthesiology* 2013; **119**:901–906.
- 26 Schlapfer M, Leutert AC, Voigtsberger S, *et al.* Sevoflurane reduces severity of acute lung injury possibly by impairing formation of alveolar oedema. *Clin Exp Immunol* 2012; **168**:125–134.
- 27 Krinke G. *The laboratory rat*. San Diego, California: Academic Press; 2000.
- 28 Urner M, Schläpfer M, Herrmann I K, *et al.* Insight into the beneficial immunomodulatory mechanism of the sevoflurane metabolite hexafluoro-2-propanol in a rat model of endotoxaemia. *Clin Exp Immunol* 2015; **181**:468–479.
- 29 Calvano SE, Coyle SM. Experimental human endotoxemia: a model of the systemic inflammatory response syndrome? *Surg Infect (Larchmt)* 2012; **13**:293–299.
- 30 Qin L, Wu X, Block ML, *et al.* Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* 2007; **55**:453–462.
- 31 Watanabe K, Kinoshita S, Nakagawa H. Purification and characterization of cytokine-induced neutrophil chemoattractant produced by epithelioid cell line of normal rat kidney (NRK-52E cell). *Biochem Biophys Res Commun* 1989; **161**:1093–1099.
- 32 Katayama T, Tanaka H, Yoshida T, *et al.* Neuronal injury induces cytokine-induced neutrophil chemoattractant-1 (CINC-1) production in astrocytes. *J Pharmacol Sci* 2009; **109**:88–93.
- 33 Johnson EA, Dao TL, Guignet MA, *et al.* Increased expression of the chemokines CXCL1 and MIP-1 $\alpha$  by resident brain cells precedes neutrophil infiltration in the brain following prolonged soman-induced status epilepticus in rats. *J Neuroinflammation* 2011; **8**:41.
- 34 Marro BS, Grist JJ, Lane TE. Inducible expression of CXCL1 within the central nervous system amplifies viral-induced demyelination. *J Immunol* 2016; **196**:1855–1864.
- 35 Deshmane SL, Kremlev S, Amini S, *et al.* Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res* 2009; **29**: 313–326.
- 36 Cazareth J, Guyon A, Heurteaux C, *et al.* Molecular and cellular neuroinflammatory status of mouse brain after systemic lipopolysaccharide challenge: importance of CCR2/CCL2 signaling. *J Neuroinflammation* 2014; **11**:132.
- 37 Andjelkovic AV, Kerkovich D, Shanley J, *et al.* Expression of binding sites for beta chemokines on human astrocytes. *Glia* 1999; **28**:225–235.
- 38 Banisadr G, Gosselin RD, Mechighel P, *et al.* Highly regionalized neuronal expression of monocyte chemoattractant protein-1 (MCP-1/CCL2) in rat brain: evidence for its colocalization with neurotransmitters and neuropeptides. *J Comp Neurol* 2005; **489**:275–292.
- 39 Boddeke EW, Meigel I, Frenz S, *et al.* Cultured rat microglia express functional beta-chemokine receptors. *J Neuroimmunol* 1999; **98**:176–184.
- 40 Ramnath RD, Ng SW, Guglielmotti A, *et al.* Role of MCP-1 in endotoxemia and sepsis. *Int Immunopharmacol* 2008; **8**:810–818.
- 41 Thompson WL, Karpus WJ, Van Eldik LJ. MCP-1-deficient mice show reduced neuroinflammatory responses and increased peripheral inflammatory responses to peripheral endotoxin insult. *J Neuroinflammation* 2008; **5**:35.
- 42 Yao Y, Tsirka SE. Monocyte chemoattractant protein-1 and the blood-brain barrier. *Cell Mol Life Sci* 2014; **71**:683–697.
- 43 Hack CE, De Groot ER, Felt-Bersma RJ, *et al.* Increased plasma levels of interleukin-6 in sepsis. *Blood* 1989; **74**:1704–1710.
- 44 Beurel E, Joep RS. Lipopolysaccharide-induced interleukin-6 production is controlled by glycogen synthase kinase-3 and STAT3 in the brain. *J Neuroinflammation* 2009; **6**:9.
- 45 Heyser CJ, Masliah E, Samimi A, *et al.* Progressive decline in avoidance learning paralleled by inflammatory neurodegeneration in transgenic mice expressing interleukin 6 in the brain. *Proc Natl Acad Sci U S A* 1997; **94**:1500–1505.
- 46 Semmler A, Frisch C, Debeir T, *et al.* Long-term cognitive impairment, neuronal loss and reduced cortical cholinergic innervation after recovery from sepsis in a rodent model. *Exp Neurol* 2007; **204**:733–740.
- 47 Rodriguez-González R, Baluja A, Veiras Del Rio S, *et al.* Effects of sevoflurane postconditioning on cell death, inflammation and TLR expression in human endothelial cells exposed to LPS. *J Transl Med* 2013; **11**:87–187.
- 48 Suter D, Spahn DR, Blumenthal S, *et al.* The immunomodulatory effect of sevoflurane in endotoxin-injured alveolar epithelial cells. *Anesth Analg* 2007; **104**:638–645.
- 49 Roy M, Richard J-F, Dumas A, *et al.* CXCL1 can be regulated by IL-6 and promotes granulocyte adhesion to brain capillaries during bacterial toxin exposure and encephalomyelitis. *J Neuroinflamm* 2012; **9**:18–118.
- 50 Mayer AMS, Murphy J, MacAdam D, *et al.* Classical and alternative activation of cyanobacterium *Oscillatoria* sp. lipopolysaccharide-treated rat microglia *in vitro*. *Toxicol Sci* 2016; **149**:484–495.
- 51 Semmler A, Okulla T, Sastre M, *et al.* Systemic inflammation induces apoptosis with variable vulnerability of different brain regions. *J Chem Neuroanat* 2005; **30**:144–157.
- 52 Norden DM, Trojanowski PJ, Villanueva E, *et al.* Sequential activation of microglia and astrocyte cytokine expression precedes increased Iba-1 or GFAP immunoreactivity following systemic immune challenge. *Glia* 2016; **64**:300–316.
- 53 Biesmans S, Acton PD, Cotto C, *et al.* Effect of stress and peripheral immune activation on astrocyte activation in transgenic bioluminescent Gfap-luc mice. *Glia* 2015; **63**:1126–1137.
- 54 Kita Y, Takashi T, Iigo Y, *et al.* Sequence and expression of rat ICAM-1. *Biochim Biophys Acta* 1992; **1131**:108–110.
- 55 Carson MJ, Thrash JC, Walter B. The cellular response in neuroinflammation: the role of leukocytes, microglia and astrocytes in neuronal death and survival. *Clin Neurosci Res* 2006; **6**:237–245.
- 56 Hoogland ICM, Houbolt C, Van Westerloo DJ, *et al.* Systemic inflammation and microglial activation: systematic review of animal experiments. *J Neuroinflammation* 2015; **12**:114.
- 57 Brahmachari S, Fung YK, Pahan K. Induction of glial fibrillary acidic protein expression in astrocytes by nitric oxide. *J Neurosci* 2006; **26**:4930–4939.
- 58 Seong K-j, Lee H-g, Kook MS, *et al.* Epigallocatechin-3-gallate rescues LPS-impaired adult hippocampal neurogenesis through suppressing the TLR4-NF- $\kappa$ B signaling pathway in mice. *Korean J Physiol Pharmacol* 2016; **20**:41–51.
- 59 Fukushima S, Furube E, Itoh M, *et al.* Robust increase of microglia proliferation in the fornix of hippocampal axonal pathway after a single LPS stimulation. *J Neuroimmunol* 2015; **285**:31–40.
- 60 Yanguas-Casás N, Barreda-Manso MA, Nieto-Sampedro M, *et al.* Tauroursodeoxycholic acid reduces glial cell activation in an animal model of acute neuroinflammation. *J Neuroinflamm* 2014; **11**:50–150.
- 61 Fink MP. Animal models of sepsis. *Virulence* 2014; **5**:143–153.
- 62 Buras JA, Holzmann B, Sitkovsky M. Animal models of sepsis: setting the stage. *Nat Rev Drug Discov* 2005; **4**:854–865.
- 63 Leibovici L. Long-term consequences of severe infections. *Clin Microbiol Infect* 2013; **19**:510–512.
- 64 Larsson JE, Wahlstrom G. The influence of age and administration rate on the brain sensitivity to propofol in rats. *Acta Anaesthesiol Scand* 1998; **42**:987–994.
- 65 Ditamo Y, Degano AL, Maccio DR, *et al.* Age-related changes in the development of experimental autoimmune encephalomyelitis. *Immunol Cell Biol* 2005; **83**:75–82.
- 66 Djikic J, Nacka-Aleksic M, Pilipovic I, *et al.* Age-associated changes in rat immune system: lessons learned from experimental autoimmune encephalomyelitis. *Exp Gerontol* 2014; **58**:179–197.
- 67 Klein SL, Flanagan KL. Sex differences in immune responses. *Nat Rev Immunol* 2016; **16**:626–638.
- 68 van Gool WA, van de Beek D, Eikelenboom P. Systemic infection and delirium: when cytokines and acetylcholine collide. *Lancet* 2010; **375**:773–775.